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COMBINATION OF CHOLINESTERASE STAINING OF NERVES AND STEREOSCOPIC VIEWING FOR THREE-DIMENSIONAL STUDY OF SKIN INNERVATION ON WHOLE MOUNTS

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A histochemical staining technique for cholinesterases is reported for the visualization of skin innervation on whole mounts in the chick. A method for realization of stereoscopic pictures showing the nerve fiber pattern in the full thickness of preparations is described. The combination of these 2 techniques allows an excellent three-dimensional demonstration of skin innervation.

The study of innervation of most structures is very difficult to accomplish in thin paraffin as well as in thick frozen sections and requires observation in the full thickness of the tissues. Therefore, different techniques were developed to visualize nerves in whole-mount preparations [1–3]. One of the main difficulties of the whole-mount method is the impossibility to obtain good photographic procedures. The present report describes a combination of cholinesterase staining of nerves in whole mounts and stereoscopic viewing. This technique was developed for the three-dimensional study of the development of skin innervation in birds.

MATERIALS AND METHODS

Cholinesterase Technique

Excision of the skin. Whole skins of the back of White Leghorn chicks from 5 days of incubation to 2 mo posthatching were used.

The embryos were decapitated and immediately placed in Tyrode solution or in 0.75% isotonic saline solution of sodium chloride. The back skin was cut in pieces as large as possible through 2 longitudinal cuts along the flanks and 2 transverse cuts at the level of neck and rump. Then using a thin curved glass rod (for 5-6 day embryos) or corneal scissors the nerves were cut close to their entering into the dorsal muscles. Afterward the whole back skin was removed. If necessary the feathers were pulled out or the calamus cut off close to the neck of the follicles.

The posthatching chickens were sacrificed with an overdose of anesthetic and the feathers rapidly plucked. Then the back skin was excised as previously described.

Cholinesterase staining. The Gomori technique for cholinesterases [4] was used with modifications as follows. The whole skin was stretched and pinned dermis up in a vessel with a wax bottom, and fixed in 10% saline formalin at room temperature for 20 min. Then the skin was carefully rinsed over a period of 30 min at 10-min intervals in 3 changes of 0.75% isotonic solution of sodium chloride. It is important at this step to remove the fat present on the dermal side, in order to secure uniform staining. Then the skin was placed in the incubation medium prepared according to Gomori. Twenty milligrams of the substrate (acetylthiocholine iodine or butyrylthiocholine iodine) per 10 ml of the Gomori stock solution were employed. Incubation for 3–4 hr at 37°C. was found correct for most specimens. The pH of the solution was between 5.6 and 6.0, which resulted in a sharp staining. After incubation the skin was rinsed rapidly in 2 changes of distilled water, immersed in 1% solution of ammonium sulfide for 2–3 min, then washed thoroughly (3-minute rinses with distilled water).

Afterward the skin was again stretched and pinned in a wax bottomed vessel and postfixed in 10% saline formalin for at least 1 hr, then dehydrated in 95% and absolute alcohol. Next the tissue was placed in a glass vessel, cleared thoroughly in 3 changes of methyl benzoate for 2 days and mounted in Canada balsam between 2 glass plates. The plates were weighted or clamped together with wooden clothespegs in order to keep the preparation flat and the whole mount was placed in an oven for at least 1 week at 50–55°C to harden the resin.

Stereomicroscopic Viewing

The stereoscopic photomicrographs were taken with an automatic Leitz Combiphot system placed on a Wild (M_4) or Leitz (Elvar) stereomicroscope. The 2 pictures (right and left) of each stereo-set (stereo-couple) of the same field of the preparation were obtained either by placing directly the camera system successively on the right and left ocular tube of the binocular microscope or by using the photographic monocular tube of the microscope equipped with a right-left built-in switching prism. With both techniques the 2 pictures of each stereo-set were taken with a 60-mm standard interpupillary distance. The stereoscopic prints shown (Fig 1–4) were mounted side-by-side with the same 60-mm point-to-point spacing, and should be observed with a stereoscopic viewer.

RESULTS AND DISCUSSION

Stereoscopic Viewing

This stereoscopic technique gives a very good demonstration of all the parts of the nerve pattern in the preparation even with very thick whole mounts (Fig 1–4). It is easy to trace the nerves throughout the skin as they travel from one layer to another.

The main disadvantage of the usual photomicrographic technique applied to thick tissue is that only the portion of the preparation in focus can be observed on the picture, so that it is necessary to take several pictures of the same area with the camera focused at different depths [3]. Although with this technique of serial pictures it is possible to build stereograms by a montage procedure [5], it is expensive, not very easy to carry out, and many details are lost.

With this very simple stereoscopic technique detailed views of full-thickness whole mounts can be easily obtained. Besides, it is also possible to get inverted stereoscopic viewing (pseudoscopy). Thus all the micrographs shown (Fig 1–4) were taken with the dermal side of the skin up, facing the lenses of the stereo-microscope and the camera. But if in the process of mounting of the stereo-couple, the right and left pictures are inverted, an artificial inversion of the three-dimensional perspective is obtained (compare Fig 3 and 4) and the image of the skin is perceived with the dermis down and the epidermis up (Fig 4).

Moreover with this technique, three-dimensional projection of stereo-couples of slides can be done using 2 projectors either with the red-and-green-filter method (method of anaglyphs) or with the polarizing filter system.

Cholinesterase Staining

This technique has given constant and useful results, and excellent demonstration of the nerve networks of the skin was achieved with a very sharp definition in whole mounts. An

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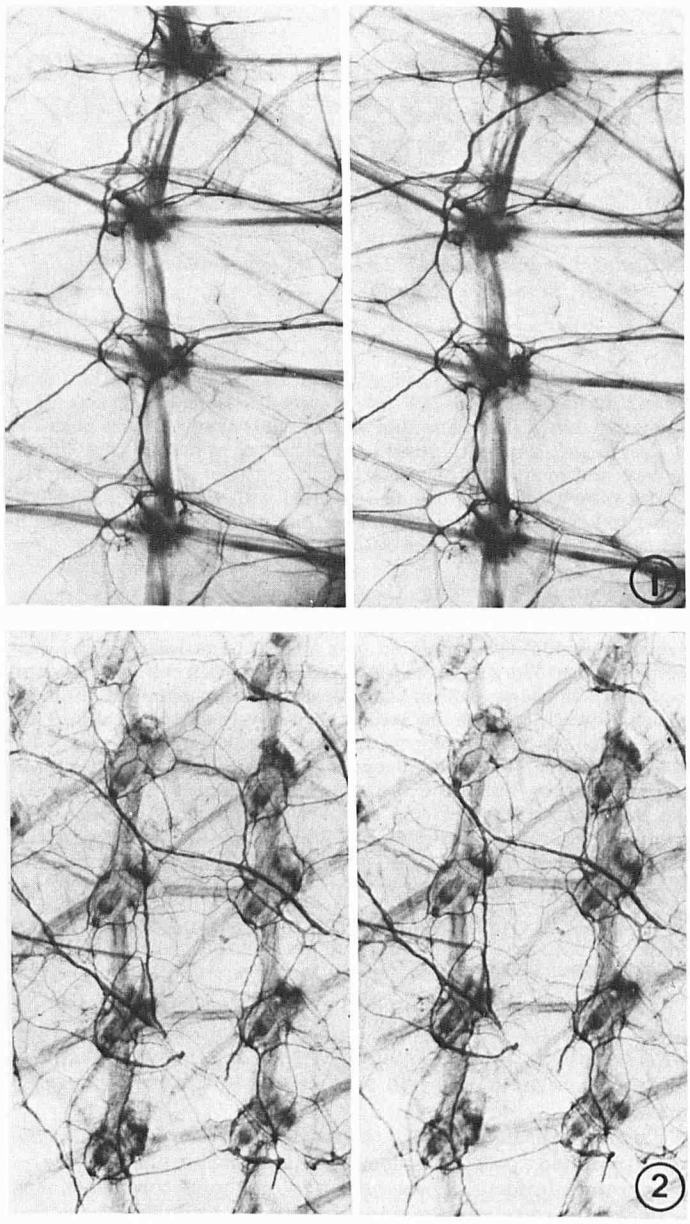

FIG 1. Stereoscopic view of the inner surface of the back skin of a 15-day posthatching chicken, oriented with the cephalic end toward top of picture, stained by the cholinesterase method as described. Four feather follicles of the dorsopelvic tracts are seen. They are interconnected by feather muscles. The pattern of innervation is seen in the full thickness of the whole mount: basal ring of nerves around each follicle, branches along the sides of the follicular sheath, innervation of the dermal papilla and feather muscles, thin nerves in the upper layers of the dermis. Some blood vessels are also seen (from top to bottom of picture: in front of the second and fourth follicles) (× 20).

FIG 2. Stereoscopic view of the inner surface of the back skin of a 16-day chick embryo, oriented with the caudal end toward top of picture. Same remarks as for Fig 1 (× 20).

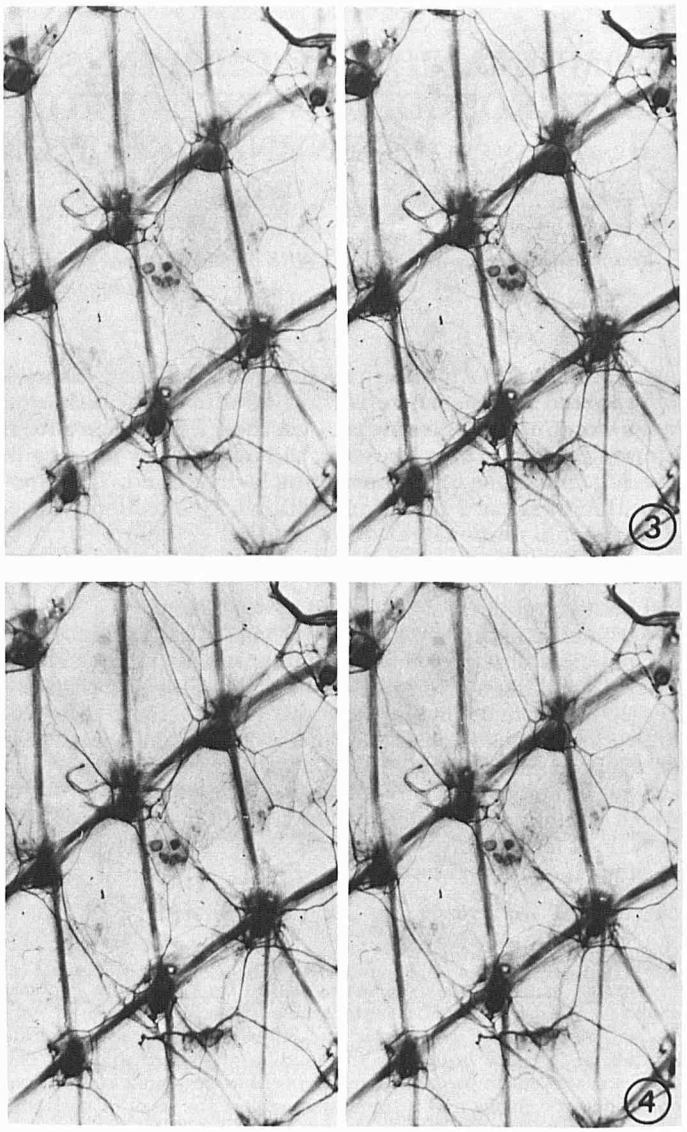

FIG 3. Stereoscopic views of the nerve network of the back skin of a 1-month-old chicken. Same remarks as Fig 1. These micrographs were taken with the dermal side of the skin-preparation up (× 15).

FIG. 4. Pseudoscopy: the right and left pictures of the above stereoset are inverted. An artificial inversion of the three-dimensional perspective is obtained, and the image of the skin is perceived with the dermis down and the epidermis up.

important advantage of the chick skin is the absence of cutaneous muscles (besides feather muscles). Indeed, when present these muscles contain diffuse cholinesterases and darken the preparation. In their absence, very thick and large specimens can be used, thus entire dorsal skins of 45-day-old chickens were stained for cholinesterases and gave excellent whole mounts measuring up to 13 × 15 cm. Another advantage of this method is that staining does not fade with time and 8-year old mounts are still in perfect state.

The validity of the histochemical results given with the Gomori cholinesterase method is well known [6] and appears to be more specific for neural tissue than the methylene blue technique, and gold chloride or silver staining [3]. However, in the case of the integument of fowl, another technique (phenylhydrazine-leucofuchsin staining) was developed by Tetzlaff, Peterson, and Ringer [2] and gave good results. The cholinesterase technique we applied to the chick skin was performed using acetylthiocholine iodine or butyrylthiocholine iodine as substrate, or in the absence of substrate. The latter control experiment (incubation without substrate) showed that the nerves were not stained. However, the structures containing a large amount of keratin (epidermis, feather calamus) had a tendency to darken the preparation, this being probably due to the action of ammonium sulfide on sulfur groups of the keratin. This darkening can be an inconvenience in the case of a very thick keratinous epidermis. In the case of incubation with butyrylthiocholine iodine substrate, no nerve network was visualized, indicating that nerves are negative for pseudocholinesterases. Therefore the staining observed in presence of acetylthiocholine iodine is due to acetylcholinesterase (that is "true" or "specific" cholinesterase).

Thus, this acetylcholinesterase method applied to chick skin (Fig 1-4) allows visualization of the arrangement of the nerve bundles and of the pattern of innervation, particularly around the follicles: basal ring of nerves, branches along the sides of the follicles, innervation of the dermal papillae. The definition allows the recognition of the behavior of small nerves and even of individual axon: thin nerves in the upper layers of the dermis, innervation of feather muscles and nerve network around the blood vessels. It is clear, then, that somatic as well as autonomic nerves can be seen with this method, as it was shown before in different organs and species [7-11] and particularly in the skin [6,12,13].

In conclusion, the demonstration of the nerve network of the chick skin in whole mounts was obtained using a cholinesterase staining method. The difficulties in photomicrographic visualization of nerves in the full thickness of the preparation, due to the too short depth of field obtained with the usual photographic techniques, were solved using stereoscopic viewing. Therefore the combination of these methods allows an excellent three-dimensional study of skin innervation.

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